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ANALYSIS OF TUMORIGENICITY AND DIFFERENTIAL GENE EXPRESSION IN FIBROBLAST CELL LINES DERIVED FROM NORMAL SKIN AND FIBROPAPILLOMAS OF THE GREEN TURTLE, (*CHELONIA MYDAS*)

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PREFACE

This report by Drs. Larry Herbst and Paul Klein presents the results of research using fibroblast cell lines derived from normal skin and fibropapilloma-type tumors of the green turtle, *Chelonia mydas*. The work was conducted in part with funds supplied by the Southwest Fisheries Science Center (SWFSC) Honolulu Laboratory's Marine Turtle Research Program. The results of previous research conducted under contracts awarded to Dr. Klein can be found in SWFSC Administrative Reports H-93-13C, H-94-10C, and H94-11C issued by the Honolulu Laboratory.

The incidence of life-threatening tumors on green turtles in the Hawaiian Islands has grown to epidemic proportions during the past decade. A similar situation exists among green turtles at certain sites in Florida, the Caribbean, and elsewhere worldwide. The cause of this disease, called fibropapillomatosis, remains unknown. However, a herpes virus has been strongly implicated. Death appears to be the usual result of the disease, although the impact to afflicted populations has not been fully assessed. The disease represents one more potentially significant threat to the survival of all green turtles. Recent findings in Florida have shown that the disease is now also occurring in increasing numbers in the loggerhead turtle, *Caretta caretta*.

The nature of fibropapillomatosis, along with its cause and mode of transmission, must be determined in order to develop a long-term management program of containment and prevention. The findings contained in the present report constitute progress in this direction which must be followed by additional research.

Because this report was prepared by independent investigators, its statements, findings, conclusions, and recommendations do not necessarily reflect the views of the National Marine Fisheries Service, NOAA.

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ABSTRACT

Fibroblast lines derived from normal skin and spontaneous or experimentally induced fibropapillomas of green turtles (*Chelonia mydas*), were established and propagated in D-MEM/F12 with 10% FBS at 30° C. Fibropapilloma derived fibroblasts were indistinguishable from normal skin fibroblasts *in vitro*. Tumor lines did not exhibit loss of contact inhibition, anchorage independence, or reduced serum requirements. Inoculation of primary and early passage tumor cells into the medial margin of the pinna of C57BL/6J-*nu/nu*, C.B17-*scid/scid*, or NOD-*scid/scid* mice, however, resulted in fibroma formation whereas inoculation of normal skin fibroblasts did not. Tumor derived cells inoculated into the flanks of mice did not form tumors. The turtle origin of fibroblasts in tumors from mouse ears was confirmed by immunohistochemistry and karyotype analysis. Fibroblast lines established from mouse ear fibromas had the normal karyotype (modal 2N = 55) of *C. mydas*. This is the first study to demonstrate a phenotypic difference between cultured fibroblasts derived from green turtle fibropapillomas (GTFP) and those from normal green turtle skin. The cooler anatomic sites (ears) of immunodeficient mice are useful for confirming the tumorigenic (transformed) phenotype of GTFP-derived fibroblasts. This mouse ear tumorigenicity test will facilitate studies of mechanisms of cellular transformation in green turtle fibropapillomatosis and other neoplastic diseases of poikilothermic vertebrates.

Preliminary studies of matched normal skin and GTFP fibroblast lines, derived from the same turtle and propagated under identical culture conditions, were performed to identify differences in messenger RNA expression between them using Reverse Transcription-Polymerase Chain Reaction Differential mRNA Display (DD RT-PCR). The tumor-derived cell line was confirmed to be tumorigenic by successful fibroma formation in mouse ears that were inoculated at the time of RNA extraction. Normal skin-derived fibroblasts inoculated at the time remained non-tumorigenic. Side-by-side comparison of the size patterns of labelled amplified cDNA sequences for 20 primer combinations revealed several clear differences, including at least 6 bands that were present in tumor but absent in normal cells, and at least 2 bands that were present only in normal cells. Four differentially amplified mRNA fragments that appeared to be overexpressed in tumor (GTFP) were cloned and their cDNA sequences were compared to available published sequences using the BLASTN search program. Two clones, LHHCM7-4 and LHHCM4-5 yielded search data which was difficult to interpret. Clone LHHCM2-2 showed homology to Bos taurus cyclin dependent kinase 1 but the overexpression of this message in the GTFP-derived line could not be confirmed by Northern Blot analysis. Clone LHHCM8-3 was homologous to human beta-hexosaminidase and the preferential expression of this sequence in the GTFP-derived cells could be confirmed. The cyclin dependent kinase would be valuable for identifying proliferating cells and the betahexosaminidase may be a useful marker for detecting internal (visceral tumors) or for tracking the progression/regression of tumors or tumor burden in GTFP-affected turtles.

Preliminary attempts to cultivate the herpesvirus associated with GTFP used preparations of transmission positive tumor extracts and cell-free filtrates containing the virus and green turtle skin fibroblasts, TH-1 *Terrapene* heart cells, a green turtle monocyte line, and VERO, a line of monkey kidney cells. Virus growth was monitored microscopically (cytopathic effects). Virus growth has not been observed to date.

KEYWORDS: Sea turtles, Green turtle fibropapillomatosis, gene expression, cell lines, herpesvirus, diagnostic tests, population health monitoring

INTRODUCTION

Populations of green turtles, *Chelonia mydas*, around the world are affected by an increasing prevalence of fibropapillomatosis, a disease that is characterized by multiple cutaneous and periocular fibroepithelial tumors and occasional visceral fibromas (1). Histologically, cutaneous green turtle fibropapillomatosis (GTFP) is characterized by benign papillary epidermal hyperplasia supported on broad fibrovascular stromal stalks (1, 2, 3, 4). Tumors are composed of well-differentiated cells with few mitotic figures and no anaplastic changes (1, 2, 4). Visceral tumors also appear to be histologically benign (1, 5, 6). The normal cytologic features of fibropapilloma cells have been corroborated by electron microscopic studies of cultured fibroblasts derived from tumors (7) and by flow cytometric analysis of cellular DNA content of tumors (8). These findings have raised the question of whether or not the extensive fibrous proliferation characteristic of these tumors represents a neoplastic or hyperplastic process (1).

Transmission experiments have implicated a subcellular transmissible agent, possibly a herpesvirus, as the cause of GTFP (9). The mechanism by which this transmissible agent causes fibroblast proliferation is unknown and may involve either direct infection and transformation of fibroblasts (neoplasia) or paracrine stimulation of uninfected fibroblasts by adjacent infected epithelium (hyperplasia).

Studies to understand the molecular pathogenesis of GTFP require the development of matched, tumor-derived and normal fibroblast cell lines and methods to distinguish benign neoplastic cells from hyperplastic normal cells. Phenotypic alterations *in vitro*, such as loss of contact inhibition (transformation foci), decreased dependence on exogenous growth factors found in serum, anchorage independence (cloning in suspension), and differences in growth rate or confluent density, can be used to detect the transformed (neoplastic) phenotype. Cellular transformation can also be detected *in vivo* by tumor formation in syngeneic animals or in an immunodeficient animal model.

This study was an attempt to demonstrate phenotypic differences between tumor-derived and normal skin-derived green turtle fibroblast lines as a first step in elucidating the pathogenesis of GTFP fibroblast proliferation. In doing so an *in vivo* system, using immunodeficient mice for testing tumorigenicity of reptile cell lines, was developed. In addition, preliminary studies of matched normal skin and GTFP fibroblast lines, derived from the same turtle and propagated under identical culture conditions, were performed to identify differences in messenger RNA expression between them using Reverse Transcription-Polymerase Chain Reaction Differential mRNA Display (DD RT-PCR). Finally, several of the cell lines and others were used in preliminary attempts to propagate the herpesvirus associated with GTFP.

MATERIALS AND METHODS

Cell Lines

Three wild green turtles with spontaneous GTFP, 11 turtles with experimentally induced GTFP (9), and one clinically normal turtle were used as sources of cell lines. Turtles were anesthetized and prepared for aseptic surgery. Fibropapillomas were resected, washed extensively in sterile saline, and minced in D-MEM/F12 with 240 U/ml Penicillin G, 240 ug/ml Streptomycin sulphate, and 0.6 ug/ml Amphotericin B (2.4 x Antibiotic-Antimycotic, GIBCO, Grand Island, NY, USA) and transported on ice to the laboratory. Multiple 6 mm punch biopsies of normal turtle skin were collected and prepared in the same way.

Tissue samples were placed in sterile 50 ml centrifuge tubes and washed 3 times in Hank's Balanced Salt Solution (HBSS, GIBCO). The HBSS was then replaced by HBSS containing 300-600 U/ml collagenase (CLS-2, Worthington Biochemical Corp., Freehold, NJ, USA) and incubated at 30°C. Disaggregated cells were harvested periodically by pipetting the digest solution into 10 volumes of ice cold D-MEM/F12 with 10% fetal bovine serum (FBS). Cells were pelleted at 400 x g for 10 minutes at 4°C and resuspended in D-MEM/F12 plus 10% FBS. Digestions were carried to completion (usually 12-24 hrs) and all cells were pooled, counted, and seeded into plastic culture flasks at a density of 1×10^5 cells per ml and incubated at 30°C in a 5% CO_2 atmosphere. Cells were grown to confluence and then collected by treating the flask with 0.25% trypsin-1 mM EDTA in HBSS. Aliquots of primary digest cells were cryopreserved in D-MEM/F12 containing 10% DMSO and 30%fetal Bovine serum. Primary cell cultures or early passage (up to TC4) were used in experiments.

In Vitro Assays

Serum dependence. The ability of cultures to grow in reduced serum concentrations was tested in two matched cell lines (TX93-A1 FP and TX93-A1 NSF). Cells from each cell line were seeded, 1×10^5 cells per well, into 6-well plates and cultured in unsupplemented D-MEM/F12 or in media supplemented with various concentrations of FBS ranging from 0.6 to 10%. Cultures were incubated at 30°C and monitored for growth. Cells were harvested and counted after 5 days, when cultures grown in 10% FBS were confluent.

Growth in agar. The ability of GTFP-derived (Coastie FP and Everglades FP) and normal skin-derived fibroblasts (Everglades NSF) to grow in soft agar was evaluated. Various concentrations ranging from 1×10^3 to 4×10^4 cells per well of each cell line were suspended in a final concentration of 0.33% agar (a 2:1 mixture of SeaPlaque® and SeaKem® agars, FMC BioProducts, Rockland, ME, USA) in D-MEM/F12 and laid over a 1% agar base in 6-well plates. Cultures were incubated at 30°C and monitored for colony formation for up to 1 month.

Transformation focus formation. Confluent cell cultures were maintained in growth medium (D-MEM/F12 plus 10% FBS) and re-fed 2-3 times a week for several weeks and observed for cell overlap and piling up.

Tumorigenicity Assay

Experiments were conducted to determine whether GTFP-derived fibroblasts could form tumors in one of the established laboratory animal models, congenitally athymic (nude, *nu*) or severe combined immunodeficiency (*scid*) mice.

Mice. C57BL/6J-*nu/nu*, C.B17-*scid/scid*, and NOD-*scid/scid* were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained in pairs on hardwood chips in polycarbonate microisolator cages (12 1/2 x 9 1/4 x 6 inches) fitted with filter tops. Mice were fed autoclaved rodent chow (Ralston Purina, St. Louis, MO, USA) and provided with sterile water containing 1 mg/ml sulfaquinoxaline (Ralston Purina, Sulfa-Nox Liquid). The mouse room was maintained on a 12:12 light cycle, temperature 21 ± 1°C.

Inoculations. In preliminary experiments, GTFP-derived cells were injected subcutaneously into the flank, footpad, and medial margin of the pinna (ear). Later experiments used ear inoculations only. Aliquots of 2×10^6 to 5×10^6 cells suspended in 100 ul PBS were injected. Mice were observed weekly for at least 4 months for evidence of tumor development. Tumors developing in mouse ears were examined by immunohistochemical methods and karyotyped to identify their species of origin.

Immunohistochemistry. Fibromas developing in mouse ears were tested with a polyclonal mouse immune serum against green turtle tissue. Briefly, 5 um sections of 10% formalin-fixed, paraffin embedded tissue were deparaffinized and rehydrated through a graded alcohol series. Sections were treated with 3% H_2O_2 to block endogenous peroxidase activity, and then treated with 0.125% trypsin-0.1% $CaCl_2$ in PBS for 20 minutes at 37°C to recover antigenicity. After washing for 30 minutes with 3 changes of PBS the sections were incubated for 2 hours with dilutions (1:100, 1:500, or 1:1000) of either normal BALB/c serum or immune serum produced in BALB/c mice by repeated inoculation with GTFP homogenate in RIBI's adjuvant. Mouse antibody binding was detected using a horseradish peroxidase conjugated avidin-biotin complex kit, following manufacturer's instructions (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA). Slides were incubated in substrate (3,3' diaminobenzidine, 0.3 mg/ml in PBS) and the color reaction monitored in control slides.

Karyotyping. Fibromas from mouse ears were collected under aseptic conditions, washed in sterile HBSS and disaggregated in collagenase as described above. Cells were seeded into culture flasks, grown to approximately 70% confluence, and submitted to the Cytogenetics Laboratory, University of Florida for karyotype determination. Harvest of metaphase cells was performed by arresting with exposure to demecolcine (Colcemid®, Sigma Chemical Co., St. Louis, MO, USA) (1 ug/ml final concentration) for 45 minutes, followed by brief enzymatic (trypsin-EDTA) release of attached cells. Cells were treated with 0.075 M KCl hypotonic solution for 20 minutes at room temperature and fixed in 3:1 methanol/acetic acid. Slides were prepared following standard chromosome spreading techniques. Chromosomes were treated with trypsin and stained with Geimsa, following standard human chromosome banding techniques, to produce a G-banding (GTG) pattern (10). Karyotypes were constructed via computer-imaging and homologous pairs were arranged in descending size order.

Detection of Changes in Gene Expression in GTFP

Preliminary studies to detect differences in gene expression between normal and tumor-derived fibroblasts were begun using differential message display (19).

Cell lines and RNA extraction. Two pairs of matched tumor and normal skin derived fibroblast cell lines (A-1 and D-1) were expanded to approximately 6 x 10^7 cells. Cells were allowed to reach confluence prior to extraction. Total RNA was extracted from cells using an extraction kit (Stratagene RNA Isolation Kit Cat #200345, Stratagene, La Jolla (CA), based on the method of Chomczynski and Sacchi (1987). Briefly, for each cell line cells were detached from their flasks with trypsin-EDTA, washed in HBSS, and counted. An aliquot of 5 x 10^6 cells of each cell line was inoculated into *scid/scid* mouse ears to confirm the phenotype at the time of RNA extraction. Aliquots containing 3 x 10^7 cells were pelleted at 500 X g for 10 minutes at 4°C. The pellets were immediately resuspended in ice cold denaturing solution (5.7 M guanidinium isothiocyanate with 7.2 mM final concentration ß-mercaptoethanol added) at a working volume of approximately 500 μl per 1 X 10^6 cells. To this suspension was added one-tenth volume of 2 M sodium acetate (pH 4.0) followed by water-saturated phenol in a volume equal to the denaturing solution volume and chloroform:isoamyl alcohol mixture equal to one-fifth the volume of denaturing solution. The suspension was then vortexed vigorously for 5 minutes and then allowed to stand on ice for 15 minutes. The mixture was then centrifuged for 20 minutes at 10,000 rpm at 4°C. The upper aqueous phase was removed with care taken to avoid the interface. Total RNA was precipitated from the aqueous phase with a volume of isopropanol at -70°C overnight. The precipitate was then pelleted and resuspended in 1/3 original volume of denaturing solution and precipitated with 1 volume of isopropanol. The precipitate was then pelleted, washed in ethanol, and dried. The pellet was resuspended in DEPC treated water to which 1/10th volume of 3 M sodium acetate (pH 5.2) was added followed by 2 x volume of 100% ethanol. The precipitate was pelleted, washed, air dried, and resuspended in 200 μl water. Recovery was monitored by the ratio of absorbances (OD_{260}:OD_{280}).

Differential message display. Total RNA extracts from 2 matched cell lines were submitted to Dr. Ratna Chakrabarty (University of Florida, Gainesville, FL) for the initial stages of differential message display (Liang & Pardee, 1993). Briefly, the RNA preparations were treated with RNAse-free DNAse to remove possible chromosomal DNA contamination. The cDNAs of a subset of total mRNA were produced by reverse transcription using 4 sets of degenerate anchored (3') primers (T12MN) where M is G, A, or C and N is G, A, T, or C. With 12 possible combinations of the last 2 bases, each primer recognizes 1/12th of the total mRNA population.

Partial cDNA sequences were amplified using 5' end primers, corresponding 3' end primers, and labelled dATP such that 50-100 cDNAs were amplified. The 5' primers were arbitrary decamers allowing annealing positions to be randomly distributed in distance from the polyA tail. The 5' primers were designed to maximally randomize the 3' end with a fixed 5' end. Following amplification, short 100-500 bp cDNA sequences were separated on a sequencing gel. The tumorigenic and normal cell products were run on adjacent lanes allowing side-by-side comparison of the mRNA expression pattern of tumorigenic versus normal skin fibroblasts. Bands that are present in one cell line but absent in the other were

normal skin fibroblasts. Bands that are present in one cell line but absent in the other were marked for further study.

Differentially expressed mRNA fragments (10 in all) were cut from the differential display (sequencing) gel and reamplified by PCR. Four differentially amplified mRNA fragments that appeared to be overexpressed in tumor (GTFP) relative to normal fibroblast cultures were selected for further analysis. These were fragments were extracted from the agarose gel matrix using a commercial kit (Qiagen, Qiaex beads) and cloned into pGEMt vector (ligated into pGEMt vector, transformation of Hanahan competent *E. Coli* and selection of transformants on Luria Broth with Ampicillin).

Sequencing. Clones (2-2, 4-5, 7-4, and 8-3) were sequenced by the dideoxynucleotide sequencing reaction. Sequences were compared to available published sequences using BLASTN program.

Northern Blotting. Extracts of total RNA (60 ug) from GTFP and normal cells were run side by side on 1% agarose RNA gel and blotted onto nitrocellulose and cross-linked with uv light. Cloned sequences to be used as Northern Blot probes were cut from the plasmid vector with restriction enzymes, separated on a 1% agarose gel (low melt), excised from the gel, and labelled with ATP^{32}. Hybridization was carried out for 72 hrs at 42° C. Autoradiographs and phosphor images were taken of Northern blots. A ribosomal RNA probe was used as a reference to quantify the intensity of probe hybridization. Northern blotting to confirm the differential mRNA expression detected by DDPCR was repeated on total RNA extracts from matched pairs of GTFP and normal skin fibroblasts from 2 turtles (A-1 and D-1).

Virus Isolation in Cell Culture

Frozen tumor extracts from 6 transmission study donors and from 3 turtles with experimentally induced tumors, that were shown by histopathology to have herpesvirus-like inclusions and several cell-free virus-positive filtrates were used to inoculate subconfluent cultures of TH-1 Terrapene heart cells purchased from the American Type Culture Collection (ATCC CCL50), green turtle normal skin fibroblasts, a turtle monocyte line (Herbst and Klein, unpublished), or VERO cells. Cultures were grown in 6 well plates or T-flasks (Costar, Cambridge, MA, USA) in Dulbecco's Modified Eagle Medium with nutrient mixture F-12 (D-MEM/F12, GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) at 25° and 30°C in a 5% CO_2 atmosphere. For infection, subconfluent cultures were washed with three changes of serum free media (D-MEM/F12). Crude tumor homogenates prepared as for transmission experiments were diluted (1:10 through 1:500) in Hank's Balanced Salt Solution (HBSS). Aliquots (500-1000 μl) were applied to each well of target cells and allowed to incubate for 30-60 minutes at 30°C to allow for attachment and cell entry. The wells were then filled with serum-free media and allowed to incubate overnight. Infected cultures were then incubated for up to 3 weeks in media supplemented either with no serum or 5-10% FBS and monitored for cytopathic effects.

RESULTS

In Vitro Characteristics of Cell Lines

Twenty four green turtle fibroblast lines were successfully established and included 12 GTFP cell lines derived from 9 cases of experimentally induced and 3 cases of spontaneous GTFP, 11 cell lines derived from normal skin of GTFP-affected turtles, and 1 cell line derived from an unaffected turtle (Table 1). There were 9 pairs of matched fibroblast lines (GTFP-derived and normal skin-derived from the same individual). Primary cell lines from some individuals failed to become established due to fungal contamination.

Cultured tumor-derived fibroblasts were morphologically indistinguishable from cultured normal fibroblasts under light microscopy and grew to similar confluent densities, approximately 4×10^4 cells/cm^2 (Fig. 1*a* and *b*). Several serum limitation experiments failed to show any differences in growth rate between normal and GTFP-derived lines that were tested (data not shown). Similarly, transformation foci were not observed and several attempts to grow cells from GTFP-derived cell lines in soft agar were unsuccessful.

Tumorigenicity of GTFP-Derived Cell Lines

Preliminary experiments, in which 2 tumor-derived cell lines were inoculated into flank, footpad, or medial ear margin of two C.B17-*scid/scid* mice, resulted in fibroma growth in the ear and footpad, but not in the flank, after at least 6 months observation. In subsequent experiments, 12 GTFP-derived fibroblast lines and 12 normal fibroblast lines were tested for tumorigenicity in the ears of 1 or more *scid/scid* or *nu/nu* mice. While none of the 12 normal skin-derived fibroblast lines caused tumors after more than 4 months observation, all tumor-derived fibroblast lines caused tumors in one or more immunodeficient mice (Table 1). Tumors (Fig. 2) were produced more reliably in *scid/scid* mice (12 of 12 lines tested, 18 of 19 mice injected) than in *nu/nu* mice (2 of 3 lines tested, 2 of 5 mice injected).

Visible tumors 1-2 mm in diameter were first noted between 5 and 44 weeks post inoculation. This range probably results from variation in actual dose of cells received and from difficulties in detecting early tumors which could be obscured by hair or skin folds. Mice inoculated with 5×10^6 cells developed tumors earlier ($x = 8 \pm 4$ weeks) than those inoculated with 2.5×10^6 cells ($x = 20 \pm 19$ weeks).

Fibromas growing in mouse ears had positive immunohistochemical reactions with mouse anti-GTFP immune serum (Fig. 3*a*) but not with normal mouse serum (Fig. 3*b*). Positive anti-GTFP staining was found in stromal cells (fibroblasts) but not in endothelium or blood cells within tumors. Normal mouse dermis and subcutis adjacent to fibromas did not show immunoreactivity (Fig. 3*a*).

Karyotypes (Fig. 4) of GTFP cell lines re-established from mouse ear fibromas were typical for *Chelonia mydas* (modal 2N = 55) (11, 12), as opposed to mouse (2N = 40), confirming the green turtle origin of these tumors.

Cloned sequences

Four unique cDNA cloned sequences were studied. These appeared to be overexpressed in the tumor by DDPCR (Differential Display). These are described below.

Clone LHHCM2-2. Clone LHHCM2-2 is a 189 bp sequence from the 3' end of GTFP mRNA, ending in a poly A tail. A BLASTN search of the sequence databases yielded weak (high score 122, prob 0.85) homology to Bos taurus cyclin dependent kinase 1 ([gb L26547] BOVRNASEQA). This result is intriguing, as cyclin dependent kinases are integral components of cell cycle regulatory apparatus. Over-expression of CDKs is associated with actively proliferating cells and may be a marker for cellular transformation. Unfortunately, the overexpression of this message could not be confirmed by Northern Blot analysis. No hybridization by labelled 2-2 clone probe could be detected on autoradiographs. This may be due to the very small size of this clone (189 bp) and/or low concentration of mRNA for this gene in the total RNA extract tested. Full length cDNA for this gene will be needed to confirm its identity and expression in GTFP versus normal cells.

Clone LHHCM4-5. Clone LHHCM4-5 is a 411 bp sequence of unknown polarity from an unknown location on the mRNA. This is because both ends of the cloned insert lacked a poly A tail to identify the 3' end of the sense strand. It is difficult to understand how this sequence was amplified in DDPCR. Database search yielded some homology to human HCG VII mRNA (emb X80916 HSHCGVII) (high score 300, prob 1.7×10^{-5}) and human TB3-1 mRNA (gb M75715 HUMTB31A) (high score 228, prob 2.0×10^{-2}). We do not know the identity or function of these human genes. It is possible that this is a false-positive result. The over-expression of this sequence in GTFP was demonstrated in Northern Blots of A-1 tumor and normal RNA.

Clone LHHCM7-4. Clone LHHCM7-4 is a 384 bp sequence with a poly A tail (from 3' end of the message). There were no homologies to the plus strand found in the BLASTN search, so we, as yet, have no idea what this gene may be. The overexpression of this message in GTFP could not be confirmed by Northern Blots due to poor/inconsistent hybridization of the probe.

Clone LHHCM8-3. Clone LHHCM8-3 is 368 bp sequence from the 3' end of the mRNA. Several homologous sequences were identified in the database. The highest scoring sequences were for human beta-hexosaminidase (gb M23294 HUMHEXB13, gb M19735 HUMBHEXB, and gb M13519 HUMHAB) (high score 195, prob 1.3×10^{-08}). Preferential expression of this sequence in the GTFP cells was confirmed by Northern Blot analysis for A-1 turtle fibroblasts.

Cultivation of the GTFP-associated herpesvirus

Several preliminary attempts to culture the GTFP-associated herpesvirus from experimentally induced tumors or transmission study donor preparations on the various cell lines were unsuccessful. No discernible cytopathic effects could be detected at 25 or 30°C. Experiments are continuing.

DISCUSSION

Tumorigenicity of green turtle fibroblast cell lines

Investigation of the molecular pathogenesis of fibroblast proliferation characteristic of GTFP depends upon the development of methods to distinguish putative transformed cells from normal cells. In this study GTFP-derived fibroblasts could not be distinguished from normal skin-derived fibroblasts by morphology and growth characteristics *in vitro*. These results corroborate a previous study (7) that described two fibroblast lines derived from green turtle fibropapillomas as having normal *in vitro* morphology, although, in that study, fibroblast lines from normal skin were not examined for comparison. This study clearly demonstrates, however, that GTFP-derived fibroblasts are qualitatively different from normal because they produce tumors *in vivo*. These findings support the hypothesis that GTFP fibroblasts are transformed (neoplastic) cells rather than normal cells responding to hyperplastic stimuli. The fact that other *in vitro* phenotypic indicators of transformation were not exhibited by GTFP fibroblasts suggests that these cells have relatively few cellular alterations. In this respect, GTFP fibroblasts resemble keloid-derived fibroblasts in humans, which are also tumorigenic in immunodeficient mice but otherwise exhibit relatively subtle differences from normal fibroblasts (13).

Congenitally athymic (nude, *nu*) and severe-combine immunodeficiency (*scid*) mice have proven to be valuable models for tumorigenicity studies of human cancers (14, 15). However, the mouse's high body temperature has made it a poor model for transplantation of poikilothermic vertebrate tissues. For example, although Manning et al. (16) were able to demonstrate engraftment of skin from several reptiles and amphibians into nude mice, the grafts were not morphologically normal, a possible result of the suboptimal thermal environment. The failure of GTFP cells to engraft in the flanks of mice in this study also supports this conclusion.

Few experimental systems have been developed for assaying tumorigenicity of lower vertebrate cells. Lucké renal adenocarcinoma cells have been cultured in the anterior chamber of the eye of *Rana pipiens* (17). Rausch and Simpson (18) developed a model system using irradiated *Anolis carolinensis* for *in vivo* tumorigenicity studies. Irradiated lizards were able to maintain grafts for up to 2 months. Limitations on husbandry conditions and the lack of "clean" barrier reared animals makes this model difficult to use. In addition, for very slow growing tumors such as GTFP, the lizards may recover their immune competence before the tumors become clinically apparent.

This study has demonstrated that immunodeficient mice can be used in tumorigenicity assays of cells derived from poikilothermic (lower) vertebrates if inoculations are performed at cooler anatomic sites such as the footpad or pinna. The mouse ear tumorigenicity model is practical because the mice can be readily incorporated into standard laboratory animal facilities with microisolator caging systems. A disadvantage of the mouse model is that it is unlikely to be useful for studies of metastasis.

The development of matched tumorigenic and normal fibroblast lines has made possible study of the molecular pathogenesis of cellular transformation in green turtle fibropapillomatosis.

Analysis of gene expression in green turtle cell lines by differential message display technology

The advent of DD RT-PCR methodology used in mRNA differential display has been instrumental in identifying possible candidate genes that enable cells to express specific phenotypes. Little, if any, information is available on cellular genes involved in oncogenesis and where genes from a poorly characterized infectious agent may play a significant role. The availability of matched tumorigenic and normal fibroblast lines made it possible to begin studies to understand the molecular pathogenesis of cellular transformation in GTFP.

Differential message display analysis was carried out to a limited extent on matched normal and GTFP derived RNA populations. Of the 4 potentially differentially expressed genes, 2 were shown to be overexpressed in tumor versus normal cells by northern blot analysis. Although additional matched sets of RNA extracts should be tested in N. Blots to confirm these results, we have preliminary evidence that the tumorigenic phenotype of GTFP cells is associated with discrete changes in gene expression. Further work is needed to determine if those sequences that failed in N. Blots are really differentially expressed.

Full length cDNA sequences must be determined for all of these clones. This will require the production of cDNA libraries from normal and GTFP fibroblasts and screening of these clones against these libraries. An alternative approach was tried using the 5' RACE procedure. Because of time constraints however, limited progress was made with this technique. Once the full cDNA sequence has been determined, database homologies can be confirmed and superior Northern Blot tests can be conducted with larger sized probes under more stringent hybridization conditions to verify the association of specific gene expression with the GTFP phenotype.

Studies of altered gene expression associated with the GTFP phenotype will help to elucidate the molecular pathogenesis of GTFP. In this preliminary study it was disappointing not to find sequences with clear homolgies to herpesvirus or other viral genes archived in the database. However, because the identified sequences are such short fragments, it remains possible that one or all of the clones already identified, belongs to the GTFP agent. Only further work will determine this.

One should remember that in this preliminary work, only a fraction of the possible primer combinations were tested to generate the differential display gel. More thorough application of additional primer sets will no doubt identify additional differentially expressed sequences, some of which may be virus (GTFP-agent) encoded messages.

Because few if any green turtle gene sequences have been described and published, isolation and sequencing of full-length cDNAs from all DDPCR products, including those that were apparently down-regulated in GTFP cells, will be of general interest. More specifically however, we have identified 2 cloned sequences with partial homologies to vertebrate

proteins that may play a role in cellular transformation. The cyclin dependent kinase 1 homologous sequence would be of value as a marker to identify proliferating cells, and could be used in future studies of the pathogenesis of virus induced transformation. The beta-hexosaminidase homologous sequence may also be a marker for the transformed phenotype and may be useful in developing a serologic test for detecting internal (visceral) tumors or in tracking the progression/regression of tumors or tumor burden in GTFP affected turtles.

With regards to beta-hexosaminidase, this enzyme has been shown to be over expressed (different isoforms also differentially expressed) in several human and rodent malignancies and in virus infections (20). To develop this serologic test, the full-length cDNA of beta-hexosaminidase needs to be incorporated into an expression vector and recombinant protein expressed in vitro, so that it can be used as antigen in the production of monoclonal antibodies for the assay.

Initial attempts to cultivate the GTFP-associated herpesvirus

Our initial failure to culture the GTFP-associated herpesvirus lines could be due to several factors. One possibility is that the cell lines or culture conditions used were not permissive for virus entry into cells or virus replication following entry. Attempts by others to culture the GTFP-associated herpesvirus have also failed to date (Gail Sherba, University of Illinois, Urbana, IL 61801, pers. comm.). Our ongoing attempts to culture this virus are using a wider range of cell lines, incubation conditions, and media formulations.

The long term objective of this research is to produce practical diagnostic tests to detect the fibropapillomatosis virus and green turtles that have been infected in the wild with this disease agent. Achieving this objective will be of direct, practical benefit to NMFS for further research and management that includes studying epidemiological aspects of the disease, and planning for disease containment and/or cure.

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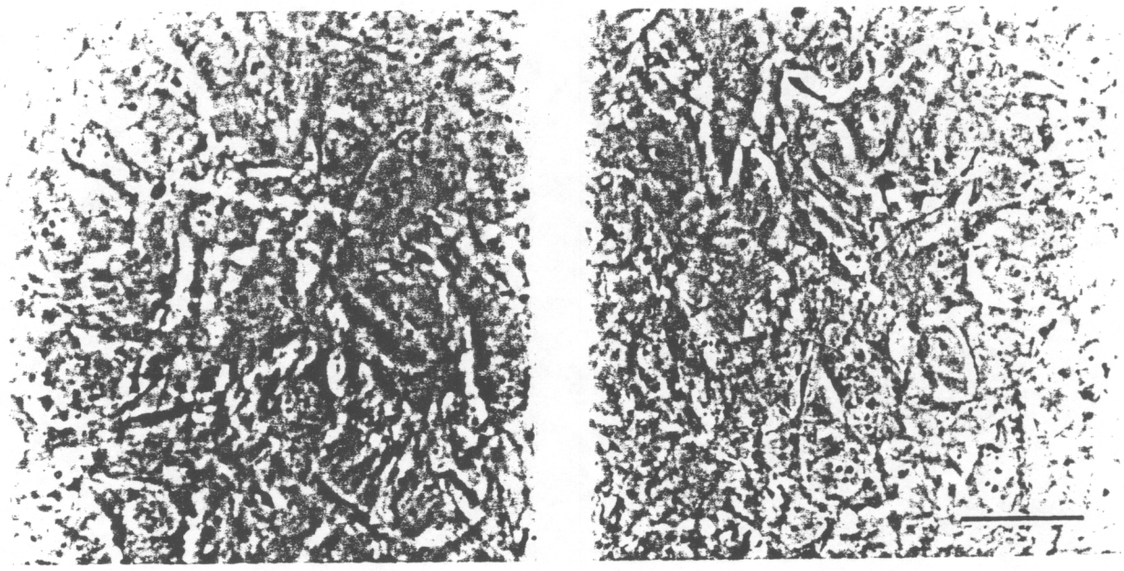
TABLE 1

TUMORIGENICITY OF FIBROBLAST LINES DERIVED FROM FLORIDA GREEN TURTLES

Cell line	Source	Tumor Production[a]		
		C.B17-*scid/scid*	NOD-*scid/scid*	C57BL/6J-*nu/nu*
TX91-#5 FP	Induced GTFP	1/1	nt[b]	nt
TX93-A1 FP	"	2/2	1/1	nt
TX93-A2 FP	"	nt	1/1	nt
TX93-A3 FP	"	nt	1/1	nt
TX93-B3 FP	"	nt	1/1	nt
TX93-C2 FP	"	nt	1/1	nt
TX93-C3 FP	"	nt	1/1	nt
TX93-D1 FP	"	1/1	1/1	nt
TX93-D2 FP	"	nt	1/1	nt
Coastie FP	Spontaneous GTFP	2/2	nt	1/3
Everglades FP	"	2/2	nt	0/1
Flamingo FP	"	2/3	nt	1/1
Jackie NSF	Normal Skin	0/3	0/1	0/3
TX93-A1 NSF	"	0/1	nt	nt
TX93-A2 NSF	"	nt	0/1	nt
TX93-A3 NSF	"	nt	0/1	nt
TX93-B2 NSF	"	nt	0/1	nt
TX93-B3 NSF	"	nt	0/1	nt
TX93-C1 NSF	"	0/1	nt	nt
TX93-C2 NSF	"	nt	0/1	nt
TX93-C3 NSF	"	nt	0/1	nt
TX93-D1 NSF	"	nt	0/1	nt
TX93-D2 NSF	"	nt	0/1	nt
Everglades NSF	"	0/1	0/1	nt

[a]Data are the number of mice with induced fibromas over the number inoculated

[b]Not tested



a b

Figure 1.--Green turtle fibroblast cultures. Confluent monolayers of early passage GTFP-derived fibroblasts (*a*) and normal skin-derived fibroblasts (*b*) from green turtle TX93-D1 grown in D-MEM/F12 with 10%FBS showing similar morphological appearance *in vitro*. Phase contrast (scale bar = 50 um).

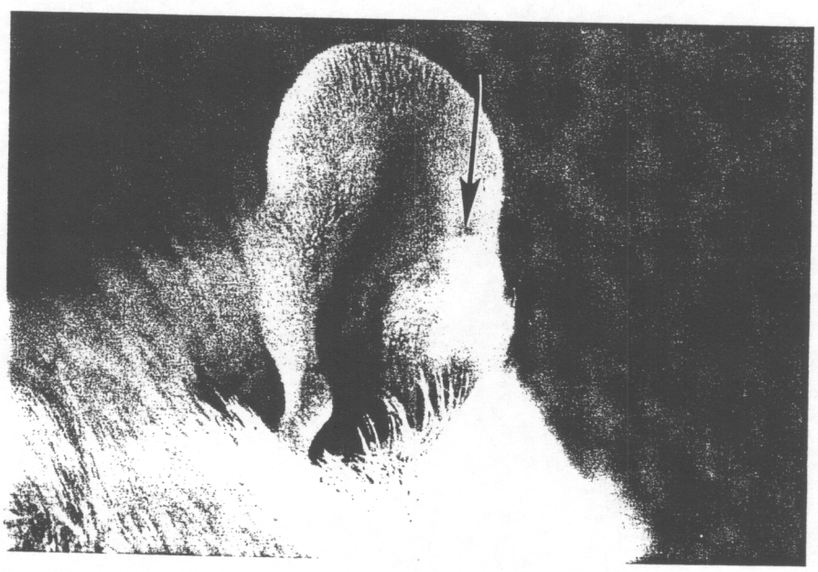


Figure 2.--Tumorigenicity of GTFP-derived fibroblasts in an NOD-*scid/scid* immunodeficient mouse. Fibroma (arrow) that developed following injection of early passage GTFP-derived fibroblasts into the medial margin of the pinna (ear).

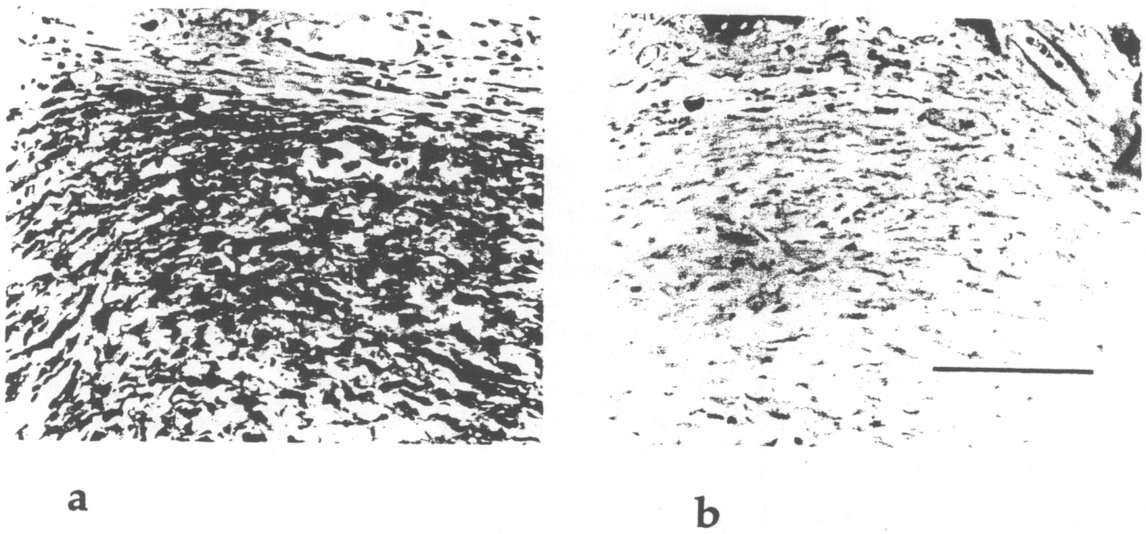


Figure 3.--Immunohistochemical detection of green turtle fibroblasts in mouse ear fibromas. Histologic sections, from fibromas that developed in mouse ears following injection of early passage GTFP-derived fibroblasts, were incubated with dilutions of serum, from BALB/c mice that were immunized with GTFP tissue homogenates, or from unimmunized mice. Binding of mouse antibodies was detected with a horseradish peroxidase-conjugated avidin-biotin complex kit. (a) Antiserum diluted 1:100 from immunized mice showed specific immunoreactivity with tumor fibroblasts but not with surrounding mouse dermis. (b) Normal mouse serum diluted 1:100 showed no specific immunoreactivity. (Scale bar = 100 um).

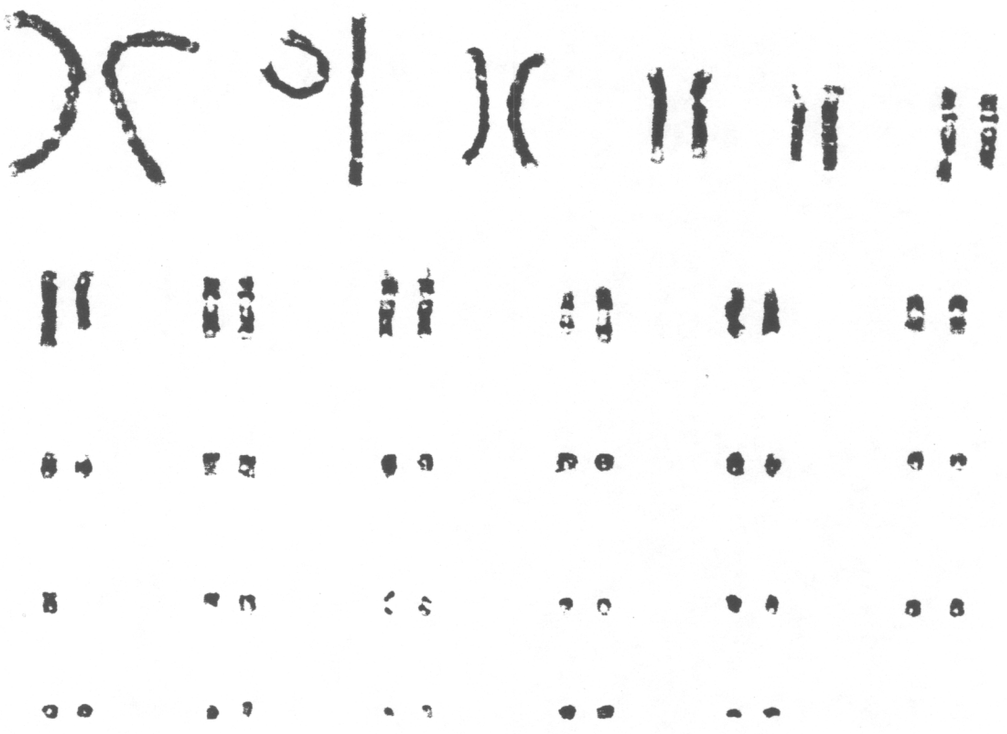


Figure 4.--Chromosomes of fibroblasts derived from tumors that developed in mouse ears following inoculation with GTFP-derived turtle fibroblasts. The karyotype (modal 2N = 55) confirmed that these cells were of green turtle origin.

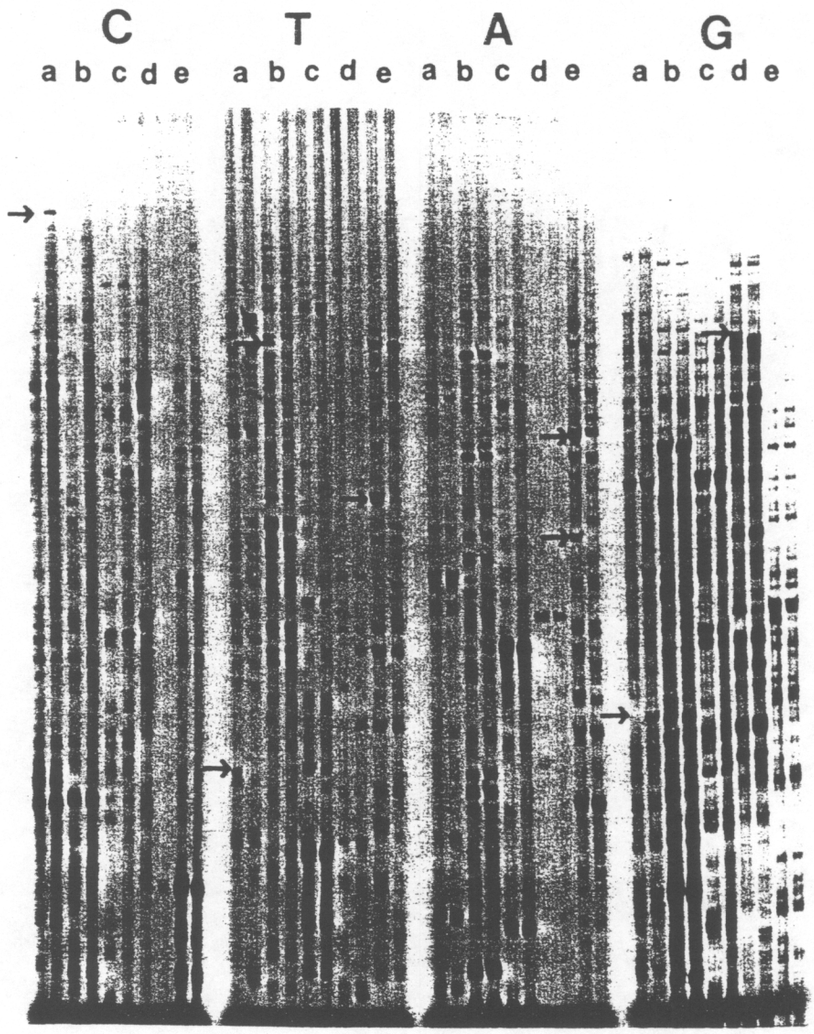


Figure 5.--Differences in gene expression between normal skin-derived and tumorigenic GTFP-derived fibroblasts. Differential message display (after 19) was used to compare the pattern of mRNA expression between two matched fibroblast lines (normal skin-derived and tumorigenic GTFP-derived) cultured from a turtle (TX93-A1) with experimentally-induced GTFP. Twenty primer combinations (4 anchored 3' primers and five 5' primers) were used to amplify, by PCR, a number of short cDNA sequences from each cell line. These short amplified sequences were labelled and separated by size on a sequencing gel. Each pair of lanes is a side-by-side comparison of amplified cDNA sequences from GTFP fibroblasts (on the left) with those from normal skin fibroblasts (on the right) for a given primer combination. The 3' primers, labelled C, T, A, or G correspond to the sequences T12CN where N is either C, T, A, or G. The 5' primers (labelled a, b, c, d, or e) were arbitrary decamers designed to have a fixed 5' end and maximally randomized the 3' end. Examples of bands that are differentially expressed are indicated by arrows.